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THE RÔLE OF PHAGOCYTOSIS IN THE PNEUMOCOCCIDAL ACTION OF PNEUMONIC BLOOD.* †

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INTRODUCTION.

OUR knowledge of the mechanism of invasion and healing in pneumococcus infections generally, and more particularly in lobar pneumonia, is as yet very incomplete. To penetrate more deeply into such mechanism and to apply newer developments in immunology, the result primarily of the researches of Wright and Douglas,[1] is the object of this investigation. This paper deals especially with phagocytosis of pneumococci *in vitro* and with the part played by phagocytosis in the pneumococcidal action of human blood, especially pneumonic, following closely the lines pursued by Ruediger[2] in his study of the streptococcidal effect of human blood, and by Hektoen[3] of the anthracidal action of dog blood.

PHAGOCYTOSIS OF PNEUMOCOCCI.

Technique.—In the phagocytosis experiments, unless otherwise specified, the technique, in the main, is that which was first worked

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[1] *Proc. Roy. Soc.*, 1903, 72, p. 357, and 1904, 73, p. 128.

[2] *Jour. Infect. Dis.*, 1906, 3, p. 156.

[3] *Ibid.*, p. 102.

out by Leishman[1] and used so extensively later by Wright and Douglas,[2] by Hektoen and Ruediger[3] and others.

Leucocytes in defibrinated or washed blood suspended in serum are used as phagocytes. The pneumococci are added either in suspensions of 0.85 per cent NaCl solution or in 24-hour broth cultures. Definite quantities of defibrinated blood or washed leucocytes, serum, and suspension of bacteria are mixed by means of graduated pipettes and then incubated at 37° C. The smears may be made at different intervals. They should be made rather thin and dried quickly, so as to spread the leucocytes over a large surface, thus making it possible to obtain accurate counts when a relatively large number of organisms are taken up. This is most conveniently done by placing on the side a large platinum loopful of the suspension and then spreading it with tissue paper cut in suitable squares. The smears are stained either by Leishman's or Wright's modification of Romanowsky's method. Both have been found equally satisfactory, although it is more difficult to prepare the former. The numbers in the tables represent the average number of pneumococci contained in each leucocyte, obtained by counting the number of bacteria in from 20 to 50 leucocytes.

Previous investigators in this field have commonly completely disregarded the influence upon phagocytosis that movement of the mixtures might have. Red blood corpuscles and leucocytes soon settle to the bottom of the tubes when kept quiet and hence the distribution of leucocytes, serum, and organisms in the mixtures necessarily becomes unequal. In order to obviate this and to approximate more closely *in vitro* the conditions present in the circulation, I devised a small shaking machine that could be placed in the incubator. With this modification in the technique phagocytosis is perceptibly increased and the results obtained would seem more reliable, because phagocytes and pneumococci are brought into closer contact. The shaking must not be too vigorous, otherwise the leucocytes are broken up. About 120 to 150 vibrations per minute with a stroke of 3 mm. is the most satisfactory. This is just sufficient to keep the corpuscles and leucocytes equally suspended and does not break up the leucocytes.

[1] *Brit. Med. Jour.*, 1902, 1, p. 73.

[2] *Loc. cit.*

[3] *Jour. Infect. Dis.*, 1905, 2, p. 128.

The effect of normal serum.—The quantitative effect of normal human serum on phagocytosis of pneumococci is shown in Table 1. As the amount of serum is diminished the number of pneumococci ingested falls until when no serum is added there is no phagocytosis.

TABLE 1.
QUANTITATIVE EFFECT OF SERUM ON PHAGOCYTOSIS.

Washed normal blood 0.2 c.c. Pneumococcal suspension 0.2 c.c.		Phagocytosis (after 1 Hour)
Normal serum 0.2 c.c.	+ NaCl sol. 0.0 c.c.	40
" " 0.1 "	+ " " 0.1 "	31
" " 0.05 "	+ " " 0.15 "	27
" " 0.025 "	+ " " 0.175 "	10
" " 0.0125 "	+ " " 0.1875 "	3
" " 0.0 "	+ " " 0.2 "	0

Variation in the susceptibility of pneumococci to phagocytosis.—The results of my early experiments quickly showed that there is great variation in the susceptibility to phagocytosis, *in vitro*, of various strains of pneumococci (Table 2).

TABLE 2.
PHAGOCYTOSIS OF PNEUMOCOCCI BY HUMAN LEUCOCYTES.

DEFIBRINATED BLOOD + PNEUMOCOCCAL SUSPENSION	NUMBER OF PNEUMOCOCCI TAKEN UP PER LEUCOCYTE IN				
	15 Min.	1 Hr.	2 Hrs.	3 Hrs.	8 Hrs.
Pneumococcus O	30	23	31	48	85
" Cl	14	20	24	25	31
" N	0.4	0.9	3.2	6	12
" S*	0	0	0	0	0.8

* This highly virulent pneumococcus was suspended in serum for three and one-half hours previous to the experiment, yet it failed to become sensitized.

Strains which have been kept upon artificial media and have lost their virulence are taken up freely (Pneumococcus O and Cl, Table 2). Others possessing a high degree of virulence for rabbits resist being taken up after 8 to 12 hours' contact with the serum (Pneumococcus S). Forty strains of pneumococci have been tested. They were obtained chiefly from the blood and sputum of pneumonia patients. All but four resisted phagocytosis at first, but usually became susceptible soon after cultivation in artificial media. In the degree they became susceptible to phagocytosis they, without exception, lose virulence.

At first smears were made at frequent intervals in order to ascertain the exact sequence of events, especially with reference to the speed with which leucocytes take up various pneumococci, whether there might be a demonstrably rapid intraphagocytic lysis of pneumococci and whether at first nonsusceptible pneumococci become so after a longer contact with opsonin. We see that in the case of Pneumococcus N (Table 2) there is practically no phagocytosis until at the end of three hours, when there is an average of six in each leucocyte; while at the end of eight hours there are 12. Is this difference between the number taken up at the end of three and of eight hours the result of an active continuous phagocytosis or the result, perhaps, of an intraphagocytic multiplication of pneumococci, or of both combined? To throw some light upon this point washed leucocytes mixed with serum and pneumococcal suspensions have been studied microscopically on the warm stage. Not only have active amœboid movements been observed so long as 12 hours, but actual ingestion of pneumococci has been seen, thus showing that the increase is, at least in part, the result of phagocytosis and not the sole result of an intraphagocytic multiplication alone. That growth may occur within the leucocytes at times can scarcely be questioned, however. In mixtures in which the organisms are taken up freely, the leucocytes first fill themselves, then burst, as it were, and finally seem to become the center of a vast colony. The question of intraphagocytic destruction of pneumococci is discussed in the later part of the paper.

EFFECT OF HEAT UPON PHAGOCYTOSIS.

Heating the serum.—Opsonin for pneumococci is considerably diminished by heating undiluted serum to 56° C. for 30 minutes, and when serum is heated to 60° for one and one-half hours there is a very marked diminution in this substance. Normal and pneumonic serum behave alike in this respect. It would thus seem that the opsonins in these sera are probably identical, and quite resistant to heat. It must be remembered, however, that the character of the organism with which one works may modify the results materially. Thus a particular pneumococcus strain which in the beginning was not at all susceptible to phagocytosis in the presence of serum, finally by long cultivation upon artificial media (seven months) became so freely taken up that carefully washed leucocytes suspended in normal salt solution took them up readily.

TABLE 3.

EFFECT OF HEATING LEUCOCYTES UPON PHAGOCYTOSIS.

Washed Blood Normal Serum Pneumococcal Suspension } Equal Parts	Phagocytosis in One Hour
Washed blood unheated	17
“ “ heated at 40° C. for 15 min.	18
“ “ “ “ 45 “ 15 “	8
“ “ “ “ 50 “ 15 “	3*
“ “ “ “ 55 “ 5 “	1.2*
“ “ “ “ 60 “ 5 “	0*
“ “ “ “ 60 “ 30 “	0

* Leucocytes surrounded by pneumococci.

Heating the pneumococci.—Heating the pneumococci has practically no effect upon their susceptibility to phagocytosis, which is in accord with the findings of Reque[1] in regard to heated diphtheria bacilli. Pneumococci of high virulence and insusceptible to phagocytosis are not made susceptible by subjecting them to 90° C. for 30 minutes.

EFFECT OF HEATING PNEUMOCOCCI ON PHAGOCYTOSIS.

Non-virulent pneumococcus, unheated	40	heated to 90° C. 30 m.	37
Slightly virulent “ “	10	“ “ 90 30	7
Highly “ “ “	0	“ “ 90 30	0

Heating the leucocytes.—When the leucocytes are heated they soon lose their phagocytic power (Table 3). The effect upon the leucocyte is the same whether one heats it in washed blood, suspended in normal salt solution, or in serum. In carrying on these experiments it was noted that when washed pneumonic leucocytes were heated side by side with washed normal leucocytes, the former were more resistant. This seems especially true of leucocytes which are obtained from cases of infection which terminate favorably.

Pneumonic leucocytes when heated at 60° C. for five minutes no longer possess any phagocytic power. Normal leucocytes usually cease to take up pneumococci at a temperature from three to five degrees lower. By the aid of the shaking machine, which serves to bring leucocytes and pneumococci in closer contact, the following interesting observation was made. When pneumonic leucocytes are heated from 50° to 60° C. for from 5 to 15 minutes and suspended in pneumonic serum, they no longer take up pneumococci, but appear markedly positively chemiotactic so that they become surrounded often by large numbers of pneumococci (see Plate 22). This frequently gives the impression of an agglutination, and suggests to the observer that the leucocyte may be the source of the agglutinin. Polymorphonuclear leucocytes only show this tendency. Normal leucocytes, heated in normal serum also do this, but to a lesser degree and at a temperature from 3° to 5° C. lower. To bring out this phenomenon well, and especially to bring out the difference in behavior of the pneumonic and normal leucocytes, the use of a shaking machine (vibrations should be about 120 per minute, with a stroke of 3 mm.) is necessary. A pneumococcus which is freely susceptible to phagocytosis and the presence of an active serum is essential to bring out this phenomenon. Leucocytes suspended in heated serum or in normal salt solution do not show this perileucocytic arrangement. Hence opsonin or some analogous substance is necessary.

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[1] *Jour. Infect. Dis.*, 1906, 3, p. 414.

That the rather remarkable difference in the behavior of the pneumonic and normal leucocytes is due to the leucocytes themselves is shown by the facts that heated normal leucocytes in pneumonic serum behave exactly the same way as in normal serum, and that pneumonic leucocytes placed in normal serum behave exactly as they do in pneumonic serum. The leucocytes from two cases of appendicitis and one case of puerperal sepsis behaved in this respect like the leucocytes of pneumonia.

OPSONIC POWER OF PNEUMONIC SERUM.

Having shown that phagocytosis of pneumococci *in vitro* is proportional to the amount of serum present, and that the opsonin in normal and pneumonic sera is probably identical, the question whether there is an actual increase of pneumococco-opsonin in pneumonia was carefully studied. Table 4 gives the results of some of the experiments on this point. It shows no appreciable increase in the opsonic powers of pneumonic serum during any part of the disease, but does show a diminution in fatal cases. Since organisms freely susceptible to phagocytosis were chiefly used with undiluted serum, these results might be explained upon the score that the number of organisms opsonized in both cases is so large that differences might not be detected. On this account strains of pneumococci of a grade of virulence such that they were taken up only in small numbers under the conditions of the experiment and dilutions of serum were used. In this way the opsonin content of the serum

TABLE 4.

THE OPSONIC POWER OF PNEUMONIC SERUM WITH RESPECT TO PNEUMOCOCCUS.

WASHED NORMAL BLOOD	2	VOL.
SERUM	2	"
PNEUMOCOCCAL SUSPENSION	1	"

DAY OF DISEASE	PHAGOCYTOSIS	
	Pneumonic Serum	Normal Serum
Day after crisis	38	41
Atypical crisis	22	20
Day after crisis	45	40
During crisis	33	43
Fifth day	32	43
Twelfth day	56	53
" " *	57	56
Sixth day	28	32
Day after crisis	23	20
Third day	28	26
Day of death, seventh	11	15
" " " "	10	12
" " " "	20	24
Day after crisis	45	40
" " "	12	10
Second day after crisis	9	6

* Homologous organism.

of three cases of lobar pneumonia was determined upon three different occasions with the result that a perceptible increase, but not a large one, over that of the normal was obtained upon the day after crisis in two of the cases. The other failed to show increase at any period. A further study of this point was undertaken by my colleague, Dr. Wolf, whose results seem to indicate that very early in pneumonia the opsonin is diminished, then it gradually increases somewhat until it reaches its maximum, usually upon the first day following crisis. At this time there is apparently a perceptible increase. Whether this increased phagocytosis in pneumonic serum is the result of an actual increase of opsonin or due to an agglutination is difficult to say, especially since pneumococcus aglutinins in pneumonia seem to be most abundant at this time, as shown in my previous paper.[1] The diminution observed very early in the disease and in the fatal cases. however, can scarcely be attributed to anything else than an actual diminution of opsonin.

COMPARATIVE PHAGOCYTIC VALUE OF NORMAL AND PNEUMONIC LEUCOCYTES.

In a long series of experiments, to be referred to later in this paper, in which defibrinated blood, normal and pneumonic, were studied side by side with reference to their pneumococcidal effect and their relative phagocytic power, it was noted that pneumonic blood containing from two to even four times as many leucocytes per cubic millimeter as the normal would give as high and even a higher degree of phagocytosis.

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[1] *Jour. Infect. Dis.*, 1904, 1, p. 280.

At first this was thought to be due to increase in opsonin in the pneumonic serum, but careful study rather negatived this idea (see Table 4), and it seemed imperative that the leucocytes be taken into account. In the literature I am unable to find detailed observations concerning physiological differences between leucocytes in infections and normal leucocytes. Metchnikoff, while he considers leucocytes in immune animals more actively phagocytic, attributes this to "stimulins" in the serum. Most investigators consider the leucocytes of infections on a par with normal leucocytes.

Care was exercised to control all possible sources of error. The samples of normal and pneumonic bloods were obtained as nearly as possible at the same time, and subjected to the same conditions of temperature. As it was essential to use washed leucocytes, the bloods were washed exactly in the same way by using large quantities of 0.85 per cent NaCl solution, mixing thoroughly each time. The washing was repeated four times. The number of leucocytes in the normal blood must be the same as those in the pneumonic blood in order that the results may be directly comparable. This was done by carefully pipetting off the proper proportion of the top layer of the centrifugated blood, rich in leucocytes, and adding the proper amount of the deep layer, containing few leucocytes, until the leucocyte counts showed an equal number in both. Table 5 shows that phagocytosis by pneumonic leucocytes is perceptibly greater than by normal leucocytes subjected to the same conditions. Strains of pneumococci which are freely taken up by both are taken up in larger numbers by the former; other strains which are more virulent and taken up to a slight extent are also taken up more freely by pneumonic leucocytes; while still other strains which are not taken up at all by normal leucocytes are taken up to some extent by pneumonic leucocytes. It is very difficult to obtain organisms of just the proper virulence to bring out this difference; numerous tests failed, but by repeated efforts the happy combination was obtained. The degree of phagocytosis in Table 5 is high in most instances, yet by making

TABLE 5.
COMPARATIVE PHAGOCYTIC VALUE OF NORMAL AND PNEUMONIC LEUCOCYTES.

WASHED NORMAL BLOOD . . . . . . 2 VOL. SERUM . . . . 2 " PNEUMOCOCCAL SUSPENSION . . . . . 1 "			WASHED PNEUMONIC BLOOD . . . . . 2 VOL. SERUM . . . . 2 " PNEUMOCOCCAL SUSPENSION . . . . . 1 "		
PHAGOCYTOSIS			PHAGOCYTOSIS		
Day Serum Was Obtained	Pneumonic Serum	Normal Serum	Day Leucocytes Were Obtained	Pneumonic Serum	Normal Serum
Day after crisis	23	22	Day after crisis	30	35
During crisis	28	26	During crisis	35	33
" "	0	0	" "	5	6.4
End of crisis	0.7	0	End of crisis	3	2
Day after crisis	23	22	Day after crisis	37	36
" before "	28	26	" before "	35	31
Seventh day, day of death	9	12	Seventh day, day of death	14	17
" " " " "	28	30	" " " " "	30	35
" " " " "	5	7	" " " " "	10	8
Fifth day	56	53	Fifth day	70	60
" "	57	56	" "	74	78

thin smears and drying them quickly, spreading out the leucocytes widely, and using a Zeiss lens the counts obtained are sufficiently accurate to be reliable. To satisfy every demand it should be stated that the same comparative results were obtained by using falling quantities of serum.

That this increased phagocytic power of pneumonic leucocytes is not due to an increase in the opsonic power of pneumonic serum alone, is certain because normal serum answers practically as well. Whether it is due to an increased motility or to an increased sensitiveness is difficult to say. In the warm stage no perceptible increased motility could be detected. This is, however, a crude test and only great differences could be made out in this way. That the higher phagocytic power of pneumonic leucocytes is due rather to the condition of the leucocytes themselves, than to increased sensitiveness to opsonic influences, is indicated by the result of the following experiments. A pneumococcus, which has been cultivated upon artificial media for over seven months, is so freely susceptible to phagocytosis that carefully washed leucocytes, suspended in normal salt solution may take up the cocci. Here, in the absence of serum, the greater phagocytic activity of the pneumonic leucocytes was also evident, and this difference cannot be explained upon the score of an increased sensitiveness to opsonic influences. Is this increased phagocytic activity of pneumonic leucocytes specific for the pneumococcus, or is it a general manifestation? It is probably a general manifestation, for the reason that leucocytes obtained from other infections, e. g., puerperal sepsis and appendicitis show practically the same increase in phagocytic power as those from the pneumonia; and is perhaps due to the preponderance of younger, and hence more active, forms.

To summarize briefly, then, I have shown: (1) That pneumonic leucocytes are more resistant to heat than normal leucocytes. (2) When heated just sufficiently to lose their phagocytic power they are more positively chemiotactic. (3) They not only take up pneumococci which are freely susceptible to phagocytosis in larger numbers, but take up strains that resist phagocytosis by normal leucocytes. (4) They are more actively phagocytic for certain rare strains of pneumococci in the absence of serum. Whether this increased activity of the leucocyte in pneumonia is more marked as the disease progresses in the favorable cases and less so in the fatal cases might be an interesting field for further study. Judging, however, from the limited number of observations I have made upon this point it seems that no such difference is demonstrable. The leucocytes in several cases that died seemed as active as those obtained before, during, or after crisis.

RELATION OF VIRULENCE AND RESISTANCE OF PNEUMOCOCCI TO PHAGOCYTOSIS.

As stated, there is a close relation between virulence and resistance to phagocytosis of pneumococci.

Cultivation of virulent pneumococci upon artificial media is generally follwed by reduction of virulence and increasing susceptibility to phagocytosis. Attempts were made to restore lost virulence and thus see whether resistance to phagocytosis also returned. At first repeated efforts failed. However, by making blood cultures from the heart's blood 12 hours after inoculation of rabbits and promptly reinoculating the isolated organism, I have been able to restore the lost virulence of a pneumococcus sufficiently so that 5 c.c. of a 24-hour broth culture proved fatal in 48 hours to a rabbit weighing 1,600 gms. In the proportion that virulence was restored, resistance to phagocytosis returned. A chronological summary of one of these experiments is given in Table 6.

It would seem that the property of virulent pneumococci which prevents phagocytosis may be restored by animal inoculations after

TABLE 6.
RELATION OF VIRULENCE AND RESISTANCE TO PHAGOCYTOSIS OF PNEUMOCOCCUS E.

Date, etc.	Phagocytosis	Rabbit Virulence of 24-Hour Broth Cultures
Oct. 30, original culture	0	1 c.c. fatal in 12 hours
Nov. 2, organism cultivated in plain broth	7	5 c.c. " " 24 "
" 13, " " " " agar	18	
" 27, same as above	33	10 c.c. not fatal
Dec. 14, organism passed through seven rabbits	3.7	5 c.c. fatal in 48 hours
Jan. 18, organism cultivated upon blood agar. Transferred only once	31	10 c.c. not fatal
Apr. 22, organism kept on blood agar without transfer for 3 mos	10.5	

it has been lost in artificial cultivation. It may be pointed out, however, that the result obtained might be due, at least in part, to a sifting-out of the non-virulent organisms which perish in the animal, while the few which survive, presumably because of greater virulence, become more and more numerous.

I have not been able to establish any definite relation between the presence of the capsule of pneumococci and virulence. It also seems that the capsule has no special influence upon the susceptibility to phagocytosis, because capsules have been demonstrated in strains which are taken up freely, and on the other hand, no capsules may be demonstrable in the case of cocci that resist phagocytosis.

In the course of the work a number of efforts were made to determine whether pneumococci isolated from the blood and not susceptible to phagocytosis in normal and heterologous pneumonic blood might not be taken up when acted on by the homologous serum. In no instance has this been demonstrable.

Just as blood-containing media conserve the virulence of pneumococci, so also they prolong the period until the recently isolated organisms become susceptible to phagocytosis. Thus, four days' growth in plain broth was sufficient to render an organism susceptible, while another organism (obtained from Dr. Wolf) was still insusceptible after daily transfers upon blood agar slants for over five months. Until recently it also possessed a rather high degree of virulence. Suspending this and the homologous organism in pneumonic serum for 24 hours rendered the former freely, and the latter moderately, susceptible to phagocytosis. The question of the absorption of pneumococco-opsonin by virulent and non-virulent pneumococci will be considered in a separate paper.

THE PNEUMOCOCCIDAL EFFECT OF NORMAL AND PNEUMONIC SERUM AND BLOOD.

In attempting to explain crisis in pneumonia a minute study of the effect upon virulent and non-virulent pneumococci of (1) the defibrinated pneumonic blood; (2) the serum, heated and unheated; (3) the washed corpuscles in normal salt solution or recombined with serum, was made, the results being controlled by analogous experiments with normal blood. A year ago I noted that while the normal and pneumonic serum and normal blood had no pneumococcidal effect, the defibrinated blood of pneumonia patients during crisis had marked effect, 0.5 c.c. destroying about 50 millions of pneumococci in four hours. Heating the defibrinated blood to 50° C. for 30 minutes destroyed the pneumococcidal effect. These results were especially interesting because the blood shortly before and soon after crisis appeared to have no such action, and it seemed as if there was present in the blood during crisis something which destroyed pneumococci. The result of one of these experiments is given in Table 7. This assumption had to be modified, however, when the same results were obtained later with leucocytotic pneumonic blood obtained before as well as after crisis. In attempting to determine the mechanism by which pneumococci are destroyed by defibrinated blood a closer study of the effects of normal and pneumonic serum heated and unheated was necessary. I have shown that normal and pneumonic serum have apparently no pneumococcidal power. When pneumococci of low vitality are used, rather large numbers must be inoculated, otherwise growth may fail.

TABLE 7.
PNEUMOCOCCIDAL EFFECT OF DEFIBRINATED PNEUMONIC BLOOD DURING CRISIS.

DEFIBRIBRINATED PNEUMONIC BLOOD 0.5 C.C.+PNEUMOCOCCI	COLONIES IN BLOOD AGAR PLATES				
	At Once	1 Hr.	3 Hrs.	10 Hrs.	24 Hrs.
During crisis	5,000	600	0	10	0
" " (heated, 50° C. 30 min.)	5,200	5,350	7,000	8,000	8,100
12 hrs. before crisis	2,750	2,500	4,500	5,500	8,500
2 days after crisis	1,500	1,250	2,500	3,500	4,500
Pneumonic serum during crisis	2,100	2,950	2,200	4.500	6,000

Table 8 shows the growth of pneumococci in normal serum. Heating the serum to 56° C. for 30 minutes has not the slightest effect

TABLE 8.
EFFECT OF NORMAL SERUM UPON PNEUMOCOCCI.

NORMAL SERUM 0.5 C.C.	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hr.	2 Hrs.	12 Hrs.	24 Hrs.
R+pneumococcus R 51*a*	4,000	4,500	4,000	5,600	4,500
R (heated)+pneumococcus R51*a*	4,200	4,000	4,000	5,400	4,600
R+pneumococcus E^2	2,000	1,700	3,580	∞	∞
R (heated)+pneumococcus E^2	2,600	2,450	5,000	∞	∞
H+pneumococcus Otto	300	50	150	5,400	∞
H (heated)+pneumococcus Otto	500	300	400	8,600	∞
R+pneumococcus E^2	1,850	1,800	2,000	6,000	300
R+hemoglobin+pneumococcus E^2	2,000	2,400	5,000	∞	∞
Control broth+pneumococcus E^2	3,800	3,000	3,500	∞	∞

upon it as a culture medium for pneumococci. The primary drop is small, and when a little hemoglobin is added it does not seem to occur.

TABLE 9.
EFFECT OF PNEUMONIC SERUM UPON PNEUMOCOCCI.

PNEUMONIC SERUM	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hrs.	3 Hrs.	12 Hrs.	24 Hrs.
3rd day+pneumococcus N	1,200	1,050	1,400	1,560	8,000
“ “ (heated *)+pneumococcus N	1,250	1,200	1,370	1,700	8,500
4th day+pneumococcus N	1,050	1,300	1,200	1,700	3,000
“ “ (heated *)+pneumococcus N	1,100	1,400	1,350	1,500	2,400
During crisis+pneumococcus E^2	2,100	1,450	3,500	6,700	∞
“ “ (heated†)+pneumococcus E^2	3,240	6,700	8,000	∞	∞
“ “ +pneumococcus O	200	110	100	2,500	4,600
“ “ (heated †)+pneumococcus O	100	95	90	1,800	3,700
Day after crisis+pneumococcus R51*a*	3,500	3,000	5,000	∞	∞
“ “ “ (heated †)+pneumococcus R51*a*	2,500	2,255	3,000	∞	8,000
4 days after crisis+pneumococcus E^2	3,240	7,000	8,000	∞	∞
10 days after crisis (heated †)+pneumococcus N	1,650	1,400	1,500	1,700	1,850
6th day+pneumococcus E^2	1,700	1,600	2,300	300	15
“ “ +hemoglobin+pneumococcus E^2	1,900	2,400	4,000	∞	∞

* 60° for one and one-half hours.

† 56° for 30 minutes.

Table 9 shows that the clear pneumonic serum, heated or unheated, is not pneumococcidal, no matter at what period of the disease it is obtained. The primary drop is somewhat more marked than in the normal serum, especially as crisis is reached and soon thereafter. This may be due to agglutination of pneumococci. Heating the serum has no effect. The addition of hemoglobin or red corpuscles materially aids the growth of the pneumococcus here also. In order to exclude all possibility of complement-amboceptor action a series of tubes, each containing 0.5 c.c. serum, were inoculated with 25 to 200 pneumococci, the whole quantity being plated in each instance.

There was usually a slight fall, then a rise in the number of organisms, just as in the experiments above. Hence the technique employed as represented in the tables is considered reliable.

Further, it was thought possible that the serum during an attack of pneumonia which terminates favorably might acquire pneumococcidal properties for the strain of pneumococci which is responsible for the infection, but have no such power for other strains. It was found, however, that pneumococci grew in the homologous sera exactly like those from other sources. In two cases the organisms were isolated from the blood the day previous to crisis and tested with the serum during crisis. In the third the organism was isolated the day after crisis and tested with the homologous serum the day following. The organisms used in these experiments were of all degrees of virulence.

The serum alone not being pneumococcidal the explanation of the destructive effect of pneumonic defibrinated blood must depend on other factors than the serum alone.

TABLE 10.
EFFECT OF NORMAL DEFIBRINATED BLOOD UPON PNEUMOCOCCI.

NORMAL DEFIBRINATED BLOOD 0.5 C.C.	COLONIES IN BLOOD AGAR PLATES				
	At Once	1.5 Hrs.	3 Hrs.	10 Hrs.	24 Hrs.
B + pneumococcus R 51*a*	1,540	1,250	150	7	0
B (heated*) + pneumococcus R 51*a*	3,000	3,500	4,500	6,000	∞
R " + " "	3,200	1,250	1,600	2,000	2,500
R " + " "	4,000	4,500	3,200	3,500	4,000
W + pneumococcus E^2	2,000	1,000	780	400	450
W (heated*) + pneumococcus E^2	2,200	1,500	3,400	∞	∞
W + pneumococcus $B1^3$ †	2,750	2,200	4,050	∞	
Washed normal corpuscles 0.2 c.c. + normal serum + pneumococcus E	1,000	900	1,150	2,500	5,000
Washed normal corpuscles (heated*) 0.2 c.c. + normal serum 0.2 c.c. + pneumococcus E	1,050	1,300	2,000	∞	∞
Washed normal corpuscles 0.2 c.c. + NaCl sol. 0.2 cc + pneumococcus E	2,600	2,850	3,000	∞	∞

* 50° C. for 30 minutes.
† Virulent organism.

Table 10 shows the effect of normal defibrinated blood upon the pneumococcus. The early reduction in the number of colonies is more marked than in the sera. Occasionally sterile plates are obtained at the end of 24 hours. The table shows further that heating the blood or the washed corpuscles, using virulent strains of pneumococci which are not susceptible to phagocytosis, and adding normal salt solution to the washed corpuscles, instead of serum, all diminish perceptibly the destructive effect.

TABLE 11.
THE PNEUMOCOCCIDAL ACTION OF PNEUMONIC DEFIBRINATED BLOOD.

DEFIBRINATED PNEUMONIC BLOOD, 0.5 C.C.	DAY OF DISEASE	LEUCOCYTOSIS	COLONIES IN BLOOD AGAR PLATES				
			At Once	1.5 Hrs.	3 Hrs.	10 Hrs.	24 Hrs.
D+pneumococcus E^2...	18 hours before crisis	21,000	2,800	400	12	6	0
D+ " E^2...	1st day after crisis	17,000	3,200	1,700	50	2	2,000
D+ " E^2...	10 days after crisis	9,000	1,890	2.100	3,500	4,000	5,000
P+ " E^2...	During crisis	27,600	2,460	1,500	400	30	2
P+ " B1^3..	" "	27,600	1,200	300	40	0	0
P+ " E....	4 days after crisis	10,000	3,640	4,200	3,500	∞	∞
S+ " E^2...	Crisis	23,000	2,300	1,000	4	25	0
S+ " B1^3..	"	23,000	1,500	200	25	0	0
V+ " S*...	5th day of disease	16,500	45	215	3,400	∞	∞
Washed pneumonic blood 0.2 c.c. + pneumonic serum 0.2 c.c.+pneumococcus E^2.........	Day before crisis	21,000	3,000	800	50	30	20
Washed pneumonic blood (heated)† 0.2 c.c. + pneumonic serum 0.2 c.c. pneumococcus E^2.	Crisis	23,000	2,400	3,100	5,600	∞	∞
Washed pneumonic blood 0.2 c.c. + NaCl sol. 0.2 c.c.+pneumococcus E^2..............	"	23,000	2,700	2,400	4,500	∞	∞

* Virulent strain.

† Heated means 50° C. for 30 minutes.

Table 11 shows that the pneumococcidal effect of pneumonic defibrinated blood is, other things being equal, proportional to the leucocytosis and apparently bears no relation to the time the blood is withdrawn—before, during, or after crisis. This agrees with the findings of Ruediger[1] in regard to the streptococcidal action of defibrinated blood. Furthermore, virulent strains grow very readily, and both living leucocytes and serum are necessary to bring about decrease in the number of colonies of non-virulent organisms.

Summary.—Normal and pneumonic sera have no pneumococcidal effect. The primary drop which occurs in serum cannot be looked upon as a true bacteriolytic action but as due rather to clumping or death of the organisms incident to change of environment. Occasionally samples of normal blood have a distinct pneumococcidal action which is due not, as first supposed, to individual differences, but to the number of leucocytes present. Pneumonic defibrinated blood is decidedly pneumococcidal, especially when the leucocytosis is high, 0.5 c.c. containing 50 millions of pneumococci may be rendered sterile in four hours.

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[1] *Jour. Am. Med. Assoc.*, 1906, 46, p. 108.

These experiments seem to indicate that while neither leucocytes nor serum alone have any pneumococcidal effect, their combination has a decided action, probably dependent on phagocytosis, and further experiments were made with a view of analyzing the mechanism involved.

TABLE 12.
PNEUMOCOCCIDAL ACTION OF HUMAN LEUCOCYTES.

MIXTURES	NO. LEUCOCYTES PER CUBIC MILLIMETER	PHAGOCYTOSIS 1 HOUR	NO. COLONIES IN BLOOD AGAR PLATES			
			At Once	1.5 Hr.	10 Hrs.	24 Hrs.
Washed corpuscles 0.5+fresh serum 0.5..	44,800	15	550	1	0	0
“ “ 0.5+ “ “ 0.5..	31,000	10	1,050	25	4	0
“ “ 0.5+ “ “ 0.5..	21,000	20	560	5	0	0
“ “ 0.5+ “ “ 0.5..	20,000	12	355	180	125	0
“ “ 0.5+ “ “ 0.5..	11,000	30	1,350	1,000	18	200
“ “ 0.5+ “ “ 0.5..	8,000	28	250	75	15	800
“ “ 0.5+ “ “ 0.5..	50	?	1,000	1,450	∞	∞
“ “ 0.5+ “ “ 0.5..	25	?	500	450	1,400	∞
Washed corpuscles (heated 50°C., 30 min.) 0.5+ fresh serum 0.5...............	44,800	0	700	625	4,300	∞
Washed corpuscles (heated 50°C., 30 min.) 0.5+fresh serum 0.5...............	11,000	0	1,250	1,300	∞	∞
Washed corpuscles 0.5+0.85% NaCl sol. 0.5..........................	28,000	0	375	320	2,400	∞
Washed• corpuscles 0.5+0.85% NaCl sol. 0.5..........................	8,000	0	1,300	1,350	∞	∞
Washed corpuscles 0.5 + fresh serum 0.5*............................	20,000	0	150	50	200	∞
Washed corpuscles 0.5+heated serum (60°C. 1.5 H.).....................	5,500	8	1,450	1,200	1,850	1,600
Fresh serum..........................	.....	.....	980	800	1,400	4,500

* Inoculated with highly virulent strain.

Table 12 is illustrative of results that have been obtained many times. The points which I wish to emphasize especially are, that the leucocyte counts were made from the tubes; that the degree of phagocytosis was obtained in parallel, but separate, tubes in which pneumococci were present in much larger numbers than in those used for study of the pneumococcidal action. The reason phagocytosis is not so high when a large as when a moderate number of leucocytes was used is due to the fact that there were not enough pneumococci present, practically all having been taken up as shown by the smears. In order to obtain sterile plates it is necessary to use organisms which are freely taken up. Normal leucocytes and serum behave practically the same as pneumonic leucocytes and serum, although the former leucocytes are not quite so active as pointed out in the foregoing. While the experiments in Table 9 show that pneumonic serum alone has no appreciable pneumococcidal effect I controlled each experiment given in Table 12 carefully by inoculating serum, so I am sure that the destruction is not the result of something in the serum which would act independently of the leucocytes.

The results shown in Table 12 establish, I believe, that the pneumococcidal effect of pneumonic and other leucocytotic blood is due entirely to phagocytosis. The following points speak for this view: The destructive effect is most marked when the degree of phagocytosis is greatest. As phagocytosis is diminished by heating the leucocytes, by heating serum, or by adding falling quantities of serum, thus diminishing the opsonin, and by using virulent pneumococci, so the pneumococcidal action fluctuates. Other things being equal the destructive action is directly proportional to the number of healthy, well-preserved leucocytes present.

Definite evidences of digestion of pneumococci within leucocytes have been made out repeatedly. In experiments with a sufficiently large number of leucocytes so that destruction of pneumococci goes on more rapidly than multiplication, the picture is striking. Smears made at intervals show not only increasingly less pneumococci per leucocyte, but also morphologic and tinctorial evidences of solution (see Plate 22). This is seen best at the end of 8 to 12 hours.

CONCLUSIONS.

Non-virulent pneumococci are freely susceptible to phagocytosis. Virulent strains are not, but in the degree in which they lose their virulence when cultivated upon artificial media they become, susceptible to phagocytosis. When lost virulence is restored, resistance to phagocytosis also returns.

Pneumonic leucocytes are perceptibly more actively phagocytic than normal leucocytes, a property which seems independent of the action of the serum. They are more resistant to heat, and when heated to the thermal death-point and resuspended in serum, they are more actively chemiotactic for pneumococci.

Pneumococco-opsonin is quite resistant to heat. In pneumonia it may fall below normal in fatal cases, while during and shortly after crisis of cases that recover it seems to rise above normal.

Pneumococci when first isolated from the blood of pneumonia patients, whether before, during, or after crisis, refuse phagocytosis *in* *vitro*, with only a few exceptions, whether exposed to the blood of the patient from which they are isolated or to blood from other sources.

The pneumococcidal action of pneumonic or other leucocytotic blood is the result of the combined action of serum and leucocytes —phagocytosis and intraphagocytic digestion. Dead leucocytes seem to have no pneumococcidal effect. Normal and pneumonic sera have no pneumococcidal action. The primary drop which often occurs cannot be looked upon as a true bacteriolysis, but as due rather to clumping or death of organisms incident to change of environment.

However important the rôle of phagocytosis in the destruction of pneumococci *in vitro*, there are still serious difficulties in the way of ascribing the crisis and healing of pneumonia to opsonification and phagocytosis alone. Thus definite experimental proof is yet lacking that the pneumococci that cause pneumonia absorb opsonin *in vivo* sufficiently to be made susceptible to phagocytosis.

I herewith wish to express my thanks to Dr. Hektoen for suggestions and aid in the work; and to the house and attending staffs of the Presbyterian Hospital for many courtesies.

EXPLANATION OF PLATE 22.

FIG. 1.—Phagocytosis of pneumococci by leucocytes in human defibrinated blood.

FIG. 2.—Attraction of pneumococci by heated leucocytes. Washed human blood was heated to 50° C. for 15 minutes, recombined with serum and placed in the shaking machine at 37° C. for 1 hour.

FIG. 3.—Digestion of pneumococci by human leucocytes in defibrinated blood. The suspension had been kept at 37° C. for 10 hours when the smear was made.

PLATE 22.

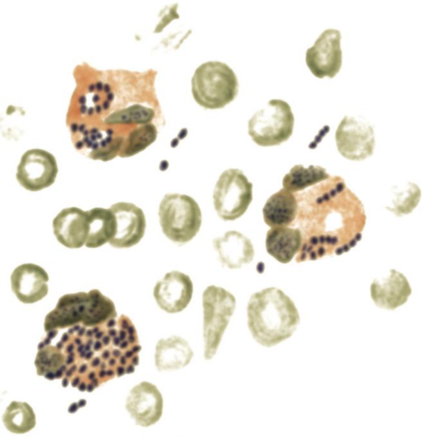

FIG. 1.

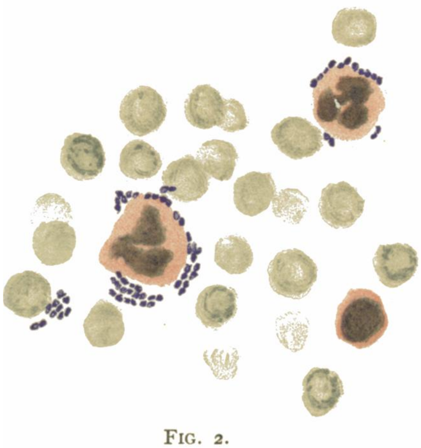

FIG. 2.

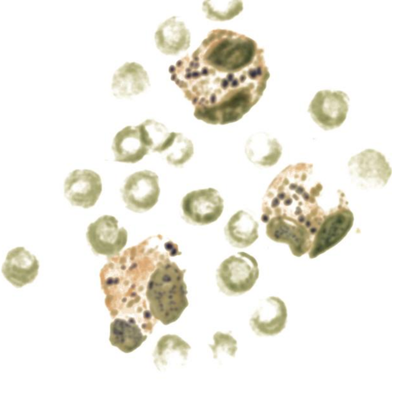

FIG. 3.